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# Regulation of nodal and BMP signaling by tomoregulin-1 (X7365) through novel mechanisms

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## Abstract

During early vertebrate development, members of the transforming growth factor beta (TGF $\beta$ ) family play important roles in a variety of processes, including germ layer specification, patterning, cell differentiation, migration, and organogenesis. The activities of TGF $\beta$ s need to be tightly controlled to ensure their function at the right time and place. Despite identification of multiple regulators of Bone Morphogenetic Protein (BMP) subfamily ligands, modulators of the activin/nodal class of TGF $\beta$  ligands are limited, and include follistatin, Cerberus, and Lefty. Recently, a membrane protein, tomoregulin-1 (TMEFF1, originally named X7365), was isolated and found to contain two follistatin modules in addition to an Epidermal Growth Factor (EGF) domain, suggesting that TMEFF1 may participate in regulation of TGF $\beta$  function. Here, we show that, unlike follistatin and follistatin-related gene (FLRG), TMEFF1 inhibits nodal but not activin in *Xenopus*. Interestingly, both the follistatin modules and the EGF motif contribute to nodal inhibition. A soluble protein containing the follistatin and the EGF domains, however, is not sufficient for nodal inhibition; the location of TMEFF1 at the membrane is essential for its function. These results suggest that TMEFF1 inhibits nodal through a novel mechanism. TMEFF1 also blocks mesodermal, but not epidermal induction by BMP2. Unlike nodal inhibition, regulation of BMP activities by TMEFF1 requires the latter's cytoplasmic tail, while deletion of either the follistatin modules or the EGF motif does not interfere with the BMP inhibitory function of TMEFF1. These results imply that TMEFF1 may employ different mechanisms in the regulation of nodal and BMP signals. In *Xenopus*, TMEFF1 is expressed from midgastrula stages onward and is enriched in neural tissue derivatives. This expression pattern suggests that TMEFF1 may modulate nodal and BMP activities during neural patterning. In summary, our data demonstrate that tomoregulin-1 is a novel regulator of nodal and BMP signaling during early vertebrate embryogenesis.

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## Introduction

TGF $\beta$  signaling has been implicated in multiple processes during early vertebrate development. Two main classes of TGF $\beta$  ligands play different roles in patterning of the early embryos (for reviews, see Hogan, 1996; Harland and Gerhart, 1997; Schier and Shen, 1999; Whitman, 2001). The activin/nodal subfamily participates in specification of endoderm and mesoderm in pregastrula embryos. At gas-

trula stages, ligands in this group are involved in dorsal mesoderm formation as well as anterior–posterior patterning. Later in development, these factors are part of the regulatory network that determines left–right asymmetry of the vertebrate body axis and functions to influence the dorsal–ventral patterning of the nervous system. Members of a second class of TGF $\beta$  ligands, the Bone Morphogenetic Proteins (BMPs), are involved mainly in ventralization of all germ layers in the early embryo, resulting in suppression of neural and dorsal mesodermal cell fates. Subsequently, BMPs participate in formation and patterning of multiple tissues and organs, including the neural crest, heart, blood,

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kidney, limb, muscle, and skeletal elements. The diverse activities of both groups of TGF $\beta$  ligands are mediated by closely related homologues in conserved signal transduction pathways, but they are also subject to differential regulation by other factors (Massague, 1998; Massague and Chen, 2000).

TGF $\beta$  signals are transduced inside cells through two types of membrane serine–threonine kinase receptors. Upon binding to ligands, type II receptors phosphorylate type I receptors (activin receptor-like kinases, or ALKs), which then activate cytoplasmic signal transducers, called Smads. Activated Smads are translocated from the cytoplasm to the nucleus and cooperate with other transcription factors to influence gene expression (for reviews, see Derynck and Feng, 1997; Massague, 1998; Massague and Chen, 2000). Though the pathway is conserved for most TGF $\beta$  members, nodal signal transduction does require an additional component, the *cripto*/*criptic*/*FRL1*/*one-eyed-pinhead* protein (Schier and Shen, 1999; Shen and Schier, 2000; Whitman, 2001). The *cripto* family proteins contain a divergent EGF domain and a conserved CFC motif, and they function as coreceptors for nodal. In the absence of *cripto*, the type I receptor ALK4 can mediate signal transduction from activin, but not from nodal (Gritsman et al., 1999). Through direct binding to both ALK4 and nodal, *cripto* allows interaction of nodal with ALK4 to stimulate downstream responses (Reissman et al., 2001; Yeo and Whitman, 2001). *Cripto* is therefore a unique component in the TGF $\beta$  pathway, which may be required specifically for nodal-related ligands.

TGF $\beta$  signals can be regulated by factors at different cellular levels. For example, the naturally occurring truncated receptor BAMBI inhibits signaling by both classes of TGF $\beta$  ligands at the membrane (Onichtchouk et al., 1999); and the inhibitory Smads, Smad6 and Smad7, block transduction of TGF $\beta$  signals in the cytoplasm (Nakao et al., 1997; Casellas and Hemmati-Brivanlou, 1998; Hata et al., 1998; Nakayama et al., 1998). The predominant regulation of TGF $\beta$  signals, however, occurs at the extracellular level. Many secreted factors have been found to antagonize the activities of TGF $\beta$  ligands in early embryos, and they include *noggin*, *chordin*, *follistatin*, *Cerberus*, *Gremlin*, *Xnr3*, and *lefty* (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995; Piccolo et al., 1996, 1999; Zimmerman et al., 1996; Hansen et al., 1997; Hsu et al., 1998; Branford et al., 2000; Cheng et al., 2000; Tanegashima et al., 2000). Interestingly, though all of these proteins can inhibit the function of BMPs, only a subset of these modulates activin/nodal activity. *Cerberus* blocks nodal, but not activin signaling through direct binding to nodal (Piccolo et al., 1999); and *lefty* has been proposed to prevent interaction of activin/nodal with their receptors by occupying these receptors (Sakuma et al., 2002). *Follistatin*, the first identified secreted factor to regulate signals from the activin/nodal group of ligands, inhibits activin but not Vg1 through specific, high affinity binding to activin

(Kogawa et al., 1991; Fukui et al., 1993). The regulation of nodal signaling by *follistatin* has not been demonstrated. While these three regulators of activin/nodal signals do not share sequence homology, several other proteins with *follistatin* domains have been recently identified. *Follistatin* contains three repetitive cysteine-rich motifs, called *follistatin* (FS) modules. Proteins containing FS modules have been identified in a variety of species. These proteins contain either a single FS module, as seen in the *follistatin*-related proteins (FRPs; Mashimo et al., 1997; Okabayashi et al., 1999; De Groot et al., 2000) and *follistatin*-like (*Flik*; Patel et al., 1996; Towers et al., 1999), or they have two or more FS modules, such as in *follistatin*-related gene (*FLRG*, which has two FS modules, Hayette et al., 1998; or *FSRP*, Schneyer et al., 2001). Like *follistatin*, *FLRG* binds to activin with high affinity and blocks activin signaling (Schneyer et al., 2001; Tsuchida et al., 2001; Bartholin et al., 2002). These data suggest that proteins with FS modules may participate in regulation of activin/nodal activities.

Recently, a transmembrane protein with two FS modules was isolated from several species (Eib and Martens, 1996; Uchida et al., 1999; Eib et al., 2000; Da Silva et al., 2001). This protein, *tomoregulin-1* (TMEFF1, previously named 7365), also contains an EGF motif in its extracellular region. The function of TMEFF1 in the regulation of TGF $\beta$  signals has not been demonstrated. In this study, we report that, unlike *follistatin*, TMEFF1 selectively inhibits nodal, but not activin in *Xenopus* ectodermal explants. Both the FS and the EGF domains contribute to the nodal inhibitory function of TMEFF1. A soluble protein containing the FS modules and the EGF motif, however, is not sufficient for nodal inhibition; membrane localization of the protein is also required. Furthermore, we show that TMEFF1 also inhibits mesodermal, but not epidermal induction by BMP2. Interestingly, the regulation of BMPs requires the cytoplasmic tail of TMEFF1; the *follistatin* modules are dispensable for this activity if the EGF motif is present, and vice versa. These results imply that different strategies may be employed by TMEFF1 to inhibit nodal and BMPs. Our data thus suggest that TMEFF1 regulates nodal and BMP signaling through novel mechanisms.

## Materials and methods

### *Construction of TMEFF1 mutants*

All mutants were constructed by using PCR-based cloning strategies. For TMEFF1- $\Delta$ C, PCR was done with TMEFF-N: GGGAATTCACCATGGATGGATTGCACCT and  $\Delta$ C-C: GGCTCGAGCTAAATACACATGACAATTGC. The PCR fragment was digested with *EcoRI* and *XhoI* and inserted into the pCS2<sup>++</sup> vector. For TMEFF1- $\Delta$ TC, PCR was performed with TMEFF-N and  $\Delta$ TC-C: GCTCTAGACTAGGTGAGCTTTTGCCTACT; the PCR product was digested with *EcoRI* and *XbaI* and

inserted into the pCS2<sup>++</sup> vector. For TMEFF1-ΔFS, PCR was done with EGF-N: CGCCACAATGGCATAGAAAC-AGATGAAACA and TMEFF-C: GGGTCTAGACTACACCATCCGGGAAGAAGT. The PCR product was cut with *Bgl*II and *Xba*I and ligated into the *Bgl*II/*Xba*I-digested pCS2<sup>++</sup>TMEFF1. For TMEFF1-ΔEGF, PCR was performed with TM-N: GAAGGCCTTTATGTGGTTCCAA-GTAGG and TMEFF-C. The PCR product was double digested with *Stu*I and *Xba*I, and ligated into the *Stu*I/*Xba*I-digested pSC2<sup>++</sup>TMEFF1. For XFS-TMEFF1, PCR was done with follistatin template with XFS-N: GCGGAATT-CACCATGTTAAATGAAAGGATC and XFS-C: GCGA-AGCTTCTTACAGTTGCAAGATCCACT. The PCR product was digested with *Eco*RI and *Hind*III. PCR was also performed with TMEFF1 template with TMEFF-EGFN: GCGAAGCTTATAGAAACAGATGAAACAA-GC and TMEFF-C. The PCR product was digested with *Hind*III and *Xba*I. The two digested PCR products were ligated and inserted into the *Eco*RI/*Xba*I sites of the pCS2<sup>++</sup>vector.

#### RT-PCR

For TMEFF1 temporal expression, the following two primers were used for RT-PCR assay: TMEFF1-U, CGAC-CCGAGGTGAAAGAT; and TMEFF1-D, GTTTTGCT-TCTGCCGTCG. Primers used for other markers are as described (Chang et al., 1997).

#### Whole-mount in situ hybridization

In situ hybridization was performed as described (Harland, 1991). The TMEFF1 probe was synthesized with T7 polymerase, using *Not*I linearized pBSKS-TMEFF1 template.

## Results

#### Selective inhibition of nodal and BMP2, but not activin, by TMEFF1

TMEFF1 is a transmembrane protein of about 370 amino acids. It has been isolated from *Xenopus*, newt, mouse, and human (Eib and Martens, 1996; De Groot et al., 2000; Eib et al., 2000; Da Silva et al., 2001). It contains two follistatin modules and an EGF motif in its extracellular domain. Since follistatin and FLRG can inhibit activin activities, and TMEFF1 contains two FS modules, we tested whether TMEFF1 could also inhibit activin. RNAs encoding TMEFF1 and activin were coinjected into the animal pole of two-cell-stage embryos. The ectodermal explants (animal caps) of injected embryos were dissected at blastula stages, and gene expression in these explants was analyzed at gastrula stages by RT-PCR. As shown in Fig. 1A, TMEFF1 does not block activin activity (lanes 3 and 4). Both meso-

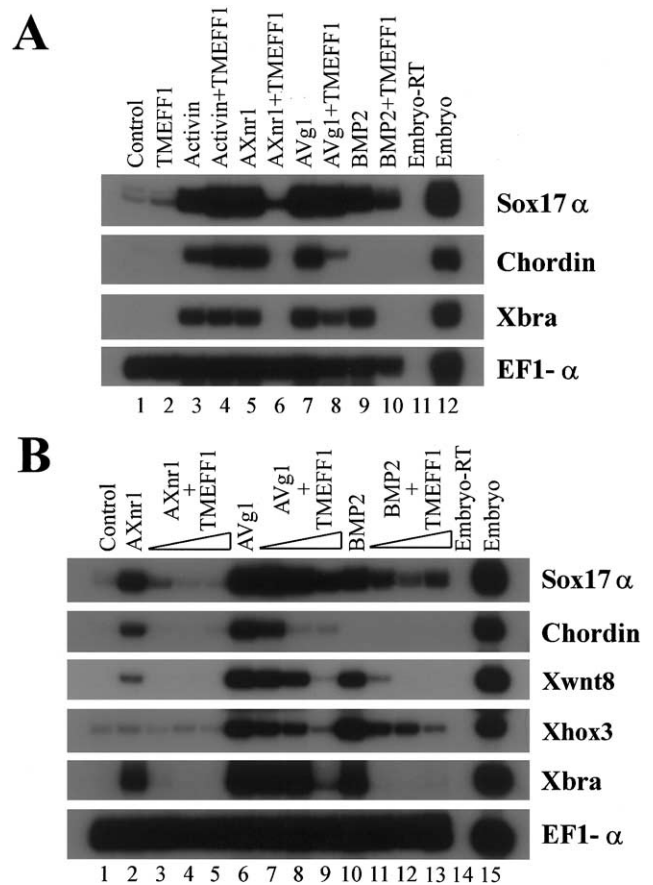


Fig. 1. TMEFF1 inhibits nodal and BMP2, but not activin, activity. (A) TMEFF1 does not inhibit mesendoderm induction by activin, but it does block nodal and reduces Vg1 and BMP2 activity. The doses of RNAs used are: activin, 5 pg; AXnr1, 0.5 ng; AVg1, 0.5 ng; BMP2, 0.5 ng; and TMEFF1, 2 ng. (B) Dose-response experiment demonstrating that TMEFF1 inhibits nodal and BMP2 efficiently, but has a weaker inhibitory activity toward Vg1. The doses of RNA used are: AXnr1, 0.5 ng; AVg1, 0.5 ng; BMP2, 0.5 ng; and TMEFF1, 0.5, 1, and 2 ng, respectively.

dermal markers, such as Brachyury (Xbra; Smith et al., 1991) and Chordin (Sasai et al., 1994), and endodermal markers, such as Sox17α (Hudson et al., 1997), are induced by activin in the presence of TMEFF1. To test the specificity of TMEFF1 for other members of the TGFβ family, we challenged the activity of nodal, Vg1, and BMP2. Chimeric nodal and Vg1 proteins, AXnr1 (Piccolo et al., 1999) and AVg1 (Kessler and Melton, 1995), containing the activin pro-domain conjugated to Xnr1 and Vg1 mature peptides, respectively, were used to ensure maximum secretion of these ligands. TMEFF1 strongly inhibits AXnr1 (Fig. 1A, lanes 5 and 6), and also blocks AVg1 and BMP2 (Fig. 1A, lanes 7–10). To examine the inhibitory efficiency of TMEFF1 toward AXnr1, AVg1, and BMP2, we performed a dose-response experiment. A fixed amount (0.5 ng) of the three TGFβ ligand RNAs was coinjected with increasing doses of TMEFF1 RNA (0.5, 1, and 2 ng) at the two-cell stage. Marker expression in animal caps from injected embryos was then analyzed at gastrula stages. As shown in Fig.

1B, TMEFF1 inhibits AXnr1 efficiently; the lowest dose of TMEFF1 is able to abolish gene induction by this ligand (Fig. 1B, compare lanes 3–5 with lane 2). TMEFF1 also effectively blocks mesodermal induction by BMP2 (Fig. 1B, compare lanes 11–13 with 10), but it displays a lower efficiency in suppressing AVg1 (Fig. 1B, compare lanes 7–9 with 6). These results suggest that TMEFF1 can regulate a subset of TGF $\beta$  ligands; however, TMEFF1 differs from follistatin in that it does not inhibit activin.

Since nodal-related ligands, but not activin, can be inhibited by the soluble antagonist Cerberus (Piccolo et al., 1999), we examined the possibility that TMEFF1 might block nodal function through induction of Cerberus in ectodermal explants. RT-PCR analysis demonstrates that Cerberus is not expressed in animal caps, and its expression is not activated by TMEFF1 (not shown). The data thus indicate that TMEFF1 may not regulate nodal signaling through this secreted nodal antagonist, though it may cooperate with Cerberus.

To further compare the ligand specificity for proteins containing FS-modules (Fig. 2A), we coexpressed follistatin (XFS), TMEFF1, and the follistatin-related gene (FLRG) with the TGF $\beta$  ligands activin, AXnr1, and AVg1 in early *Xenopus* embryos. Animal caps from injected embryos were removed at blastula stages, and gene expression patterns were analyzed at gastrula (Fig. 2B) or tailbud (Fig. 2C) stages by RT-PCR. As shown in Fig. 2, these three FS-motif-containing proteins display different specificities toward TGF $\beta$  ligands. FLRG is a specific antagonist of activin and does not interfere with mesoderm induction by AXnr1 and AVg1 (Fig. 2B and C, compare lanes 8, 12, and 16 with lanes 5, 9, and 13, respectively). While follistatin inhibits activin completely, it modifies mesoderm induction by AXnr1 and AVg1, changing the character of the mesoderm toward more dorsal fates. At gastrula stages, ventral mesodermal markers induced by AXnr1 and AVg1, such as Xwnt8 and Xhox3, are suppressed by follistatin; dorsal mesodermal and endodermal markers, such as chordin and mixer, are enhanced (Fig. 2B, compare lanes 11 and 15 with lanes 9 and 13, respectively). At tailbud stages, the axial mesodermal marker, type II collagen, which is expressed in the notochord, is induced by these ligands in the presence of follistatin, while the paraxial muscle marker, muscle actin, is inhibited (Fig. 2C, compare lanes 11 and 15 with lanes 9 and 13, respectively). Follistatin also induces neural tissues, as the expression of the neural marker NRP-1 is stimulated in the caps at tailbud stages (Fig. 2C). Dorsalization of mesoderm and neural induction by follistatin likely reflect the ability of follistatin to block BMP activities in addition to activin (Iemura et al., 1998). Consistent with the results shown in Fig. 1, TMEFF1 does not inhibit activin, but it does suppress gene activation by AXnr1 and AVg1 (Fig. 2B and C). Our data indicate that three proteins with follistatin modules have differential function in the regulation of TGF $\beta$  signaling.

Interestingly, though TMEFF1 inhibits mesoderm induc-

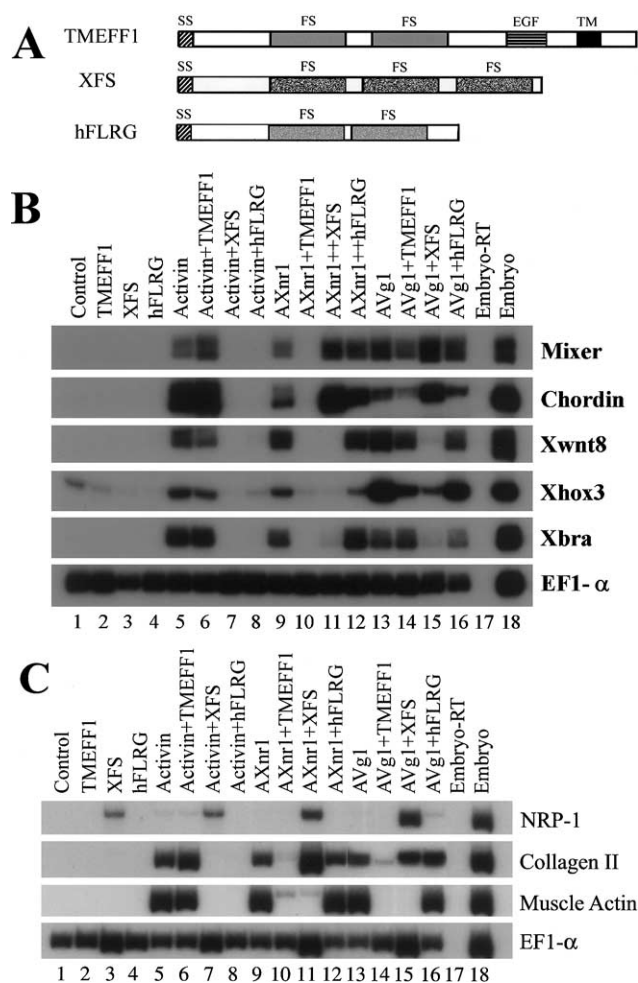


Fig. 2. Differential regulation of TGF $\beta$  ligands by follistatin-module-containing molecules. (A) Schematic representation of the three FS module-containing proteins. SS, signal sequence; FS, follistatin modules; EGF, EGF domain; TM, transmembrane region. (B, C) Follistatin (XFS), FLRG, and TMEFF1 display differential inhibitory spectrums over TGF $\beta$  ligands. While both XFS and FLRG inhibit activin, TMEFF1 does not affect mesoderm induction by activin. TMEFF1, however, blocks nodal and Vg1 function. The doses of RNAs used are: TMEFF1, 1 ng; follistatin (XFS), 1 ng; human FLRG, 1 ng; activin, 5 pg; AXnr1, 500 pg; AVg1, 500 pg; BMP2, 500 pg. The RNAs were injected into the animal region of two-cell-stage embryos, and the animal caps were dissected at blastula stages (stage 9). The caps were collected at gastrula stages (stage 11, B) or tailbud stages (stage 28, C) for RT-PCR assays of gene expression patterns.

tion by BMP2 (Fig. 1), it does not induce neural markers in animal caps (Fig. 2C). TMEFF1, however, does induce markers for cement gland, a structure outside of the anterior neural tissue whose formation requires partial inhibition of BMPs (Wilson et al., 1997; data not shown). This result suggests that TMEFF1 may be a weak inhibitor of the epidermal-inducing activity of the BMPs. To test this hypothesis, we performed a cell dissociation assay. RNAs encoding BMP2 and different doses of TMEFF1 were injected into animal poles of two-cell-stage embryos. At blastula stages, the animal caps were removed from injected embryos and dissociated in calcium- and magnesium-free

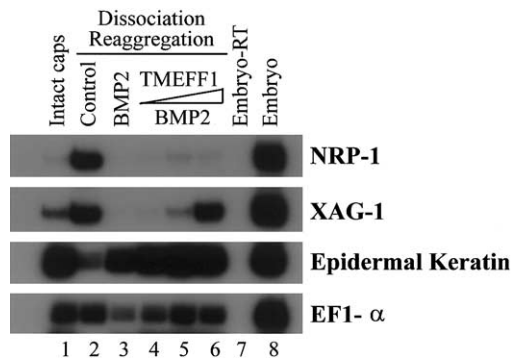


Fig. 3. TMEFF1 does not block epidermal induction by BMP2. Animal caps from control or injected embryos were dissociated at blastula stages (stage 9) for 4 h before reaggregation and incubation to tailbud stages (stage 22), at which time the RNA was extracted for RT-PCR analysis of marker expression. RNAs used for injection are: BMP2, 0.5 ng; and TMEFF1, 0.5, 1, and 2 ng, respectively.

buffer for 4 h. The cells were then aggregated and incubated to early tailbud stages for RT-PCR analysis of epidermal and neural markers. As reported before (Wilson et al., 1997), prolonged cell dissociation followed by reaggregation changes cell fate from epidermal to neural, so that epidermal keratin expression is down-regulated and the expression of the neural marker NRP-1 is activated (Fig. 3 compare lane 2 with lane 1). BMP2 restores the epidermal fate and inhibits neural formation in dissociated caps (Fig. 3, lane 3). Increasing doses of TMEFF1, while sufficient to block mesoderm induction by BMP2 (Fig. 1B), cannot reverse epidermal induction by BMP2 (Fig. 3, compare lanes 4–6 with lane 3). The data suggest that TMEFF1 differentially regulates the mesodermal- and epidermal-inducing activities of the BMPs (see Discussion).

#### *Membrane localization of TMEFF1 is required for its nodal-inhibiting activity*

Both follistatin and FLRG inhibit activin by directly binding to the ligand, preventing interaction with the activin receptors (Kogawa et al., 1991; Fukui et al., 1993; Tsuchida et al., 2001; Bartholin et al., 2002). The follistatin modules in these proteins are likely to play an important role in ligand binding. We therefore tested whether the follistatin modules in tomoregulin-1 were sufficient for nodal inhibition. A mutant TMEFF1 that contains only the two follistatin motifs (TMEFF1-FS; Fig. 4A) was made and coexpressed with AXnr1 in early *Xenopus* embryos. TMEFF1-FS is not able to block AXnr1 activity in animal caps: all markers induced by AXnr1 are still expressed in the presence of this mutant (Fig. 4B, compare lane 8 with lane 6). The data suggest that additional sequences are required for the nodal inhibitory function of TMEFF1. To see whether the extracellular domain, which contains both the follistatin and EGF motifs, is sufficient for nodal inhibition, we constructed a mutant protein with a C-terminal

deletion that eliminates the transmembrane region and the cytoplasmic tail (TMEFF1-ΔTC; Fig. 4A). This mutant, TMEFF1-ΔTC, can be secreted from oocytes injected with its RNA, suggesting that it is a secreted protein (Fig. 4C). Coexpression of TMEFF1-ΔTC with AXnr1 in ectodermal explants shows that, like TMEFF1-FS, TMEFF1-ΔTC does not block gene activation by AXnr1 (Fig. 4B, lane 9). This result demonstrates that secreted TMEFF1 does not inhibit nodal function and that TMEFF1 may need to be located at the membrane to function as a nodal inhibitor.

TMEFF1 from all species contains a conserved cytoplasmic tail, which may be important for nodal inhibition (Eib and Martens, 1996; Uchida et al., 1999; Eib et al., 2000; Da Silva et al., 2001). To see whether the cytoplasmic region is required for TMEFF1 function, we constructed a mutant TMEFF1 that has deletion in the cytoplasmic domain right after the transmembrane region (TMEFF1-ΔC; Fig. 4A). Unlike TMEFF1-ΔTC, injection of TMEFF1-ΔC into oocytes does not generate detectable protein from conditioned medium (Fig. 4C), suggesting that the transmembrane region is sufficient to retain the protein in cells. Coexpression of TMEFF1-ΔC with AXnr1 shows that this mutant possesses nodal inhibitory activity (Fig. 4B, lane 10). Our data therefore demonstrate that, unlike the soluble proteins follistatin and FLRG, the membrane localization of TMEFF1 is essential for its function.

#### *Both the FS modules and the EGF motif contribute to nodal inhibition*

Since soluble TMEFF1 is not sufficient for nodal inhibition and membrane localization is required for TMEFF1 function, it is possible that TMEFF1 interacts with membrane proteins that are involved in nodal signal transduction. This interaction may require only the follistatin modules or the EGF motif; alternatively, both domains may be required. To examine whether the follistatin modules and the EGF domain are required for tomoregulin-1 activity, we constructed two additional mutant proteins that have either the EGF or the FS domains removed, respectively (TMEFF1-ΔEGF and TMEFF1-ΔFS; Fig. 5A). As shown in Fig. 5B, deletion of either the FS modules or the EGF motif reduces the ability of TMEFF1 to block nodal function (compare lanes 4 and 5 with lane 3). The data show that, in addition to the follistatin modules, the EGF motif also contributes to the nodal-inhibiting activity of TMEFF1. Our data thus suggest that TMEFF1 inhibits nodal through a novel mechanism.

#### *Inhibition of nodal activity by a follistatin–TMEFF1 chimeric protein*

The follistatin modules have been proposed to mediate the TGFβ-regulatory activities of FS module-containing proteins. The differential regulation of activin and nodal by follistatin and TMEFF1 may therefore depend on sequence

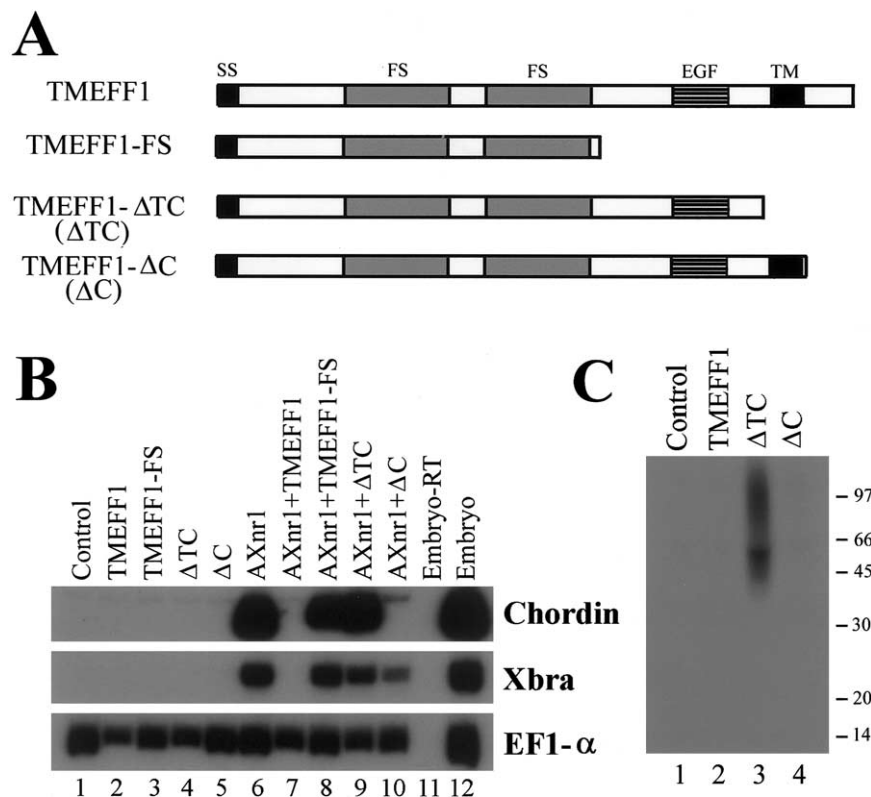


Fig. 4. Membrane localization of TMEFF1 is required for its nodal-inhibiting function. (A) Schematic representation of the deletion mutants used in this study. SS, signal sequence; FS, follistatin modules; EGF, EGF domain; TM, transmembrane region. (B) Membrane location of TMEFF1 is important for nodal inhibition. Neither the mutant protein containing the FS modules alone nor the secreted protein containing the entire extracellular domain of TMEFF1 is sufficient to block nodal function. Deletion of the cytoplasmic tail, however, does not abolish the inhibitory activity of TMEFF1. RNA (2 ng) was injected for TMEFF1 and all mutants, and 500 pg of AXnr1 was used. (C) The mutant containing the extracellular domain only (ΔTC), but not the mutant with the cytoplasmic tail deletion and intact transmembrane region (ΔC), is secreted into the medium from injected oocytes. RNA (40 ng) was used for all TMEFF1 constructs in oocyte injections. The injected oocytes were incubated in the presence of <sup>35</sup>S-methionine-labeling mix, and the conditioned supernatants were collected 2 days after injection and run on SDS-PAGE.

differences in their follistatin motifs. To examine this possibility, we constructed a chimeric XFS-TMEFF1 protein, in which the FS modules of TMEFF1 are replaced with the FS modules of follistatin (Fig. 6A). We coexpressed XFS-TMEFF1 with either activin or AXnr1 in early *Xenopus* embryos and analyzed gene expression induced by these ligands in the presence of the chimeric protein. Interestingly, as shown in Fig. 6B, XFS-TMEFF1 not only retains the nodal inhibitory activity of TMEFF1, but it also acquires the activin inhibitory function of follistatin. This result suggests that the FS modules in TMEFF1 may not be critical in the determination of ligand specificity. The data again imply that TMEFF1 regulates nodal function through a novel membrane-dependent mechanism.

#### Regulation of BMP activity by TMEFF1 requires its cytoplasmic tail

Since TMEFF1 not only inhibits nodal, but also blocks mesoderm induction by BMP2 (Fig. 1), we wanted to examine whether inhibition of BMP2 by TMEFF1 also requires the membrane location and both the FS and the EGF

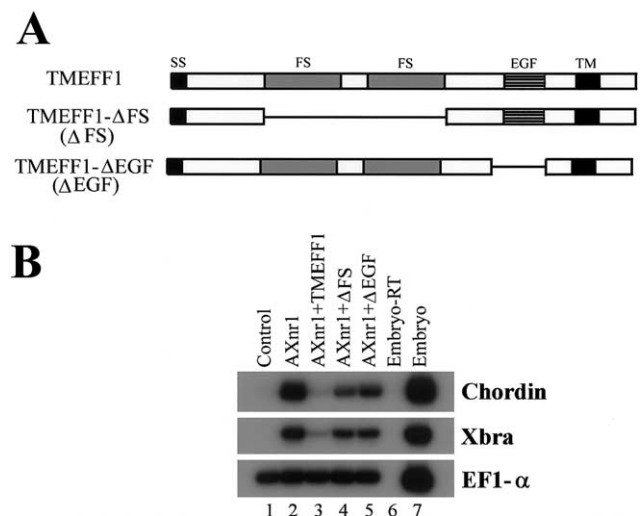


Fig. 5. Both the FS modules and the EGF motif contribute to nodal inhibition. (A) Schematic representation of the mutant proteins used. (B) Deletion of either the follistatin modules or the EGF domain reduces the ability of TMEFF1 to block nodal. The doses of RNA used in this experiment: AXnr1, 500 pg; and TMEFF1 and all deletion mutants, 2 ng.

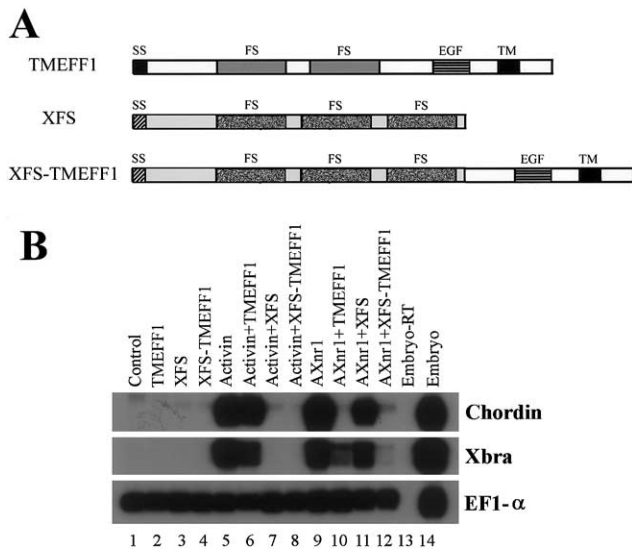


Fig. 6. The FS modules in TMEFF1 do not determine ligand specificity. (A) Schematic representation of the chimeric protein constructed. In XFS-TMEFF1, the FS modules of TMEFF1 are replaced with the FS modules of XFS. (B) The chimeric protein XFS-TMEFF1 inhibits both activin and nodal activities. The doses of RNA used are: activin, 5 pg; AXnr1, 500 pg; TMEFF1, 2 ng; XFS, 2 ng; and XFS-TMEFF1, 2 ng.

domains. We therefore coexpressed BMP2 with different deletion mutants of TMEFF1 and assayed for marker expression in animal caps from injected embryos at gastrula stages. Interestingly, neither the soluble TMEFF1 ( $\Delta$ TC) nor the membrane protein with the extracellular domain and transmembrane region of TMEFF1 ( $\Delta$ C) inhibits BMP2 efficiently (Fig. 7, compare lanes 4 and 5 with lane 3). This result implies that, unlike regulation of nodal, inhibition of BMP2 requires the cytoplasmic tail of TMEFF1. In addition, deletion of either the follistatin modules ( $\Delta$ FS) or the EGF motif ( $\Delta$ EGF) does not impair the BMP inhibitory function of TMEFF1 (Fig. 7, compare lanes 7 and 8 with lane 3). Our results suggest that TMEFF1 may use different mechanisms to block nodal and BMP signals, and inhibition of BMP by TMEFF1 may involve intracellular signaling downstream of TMEFF1.

#### Overexpression of TMEFF1 in early *Xenopus* embryos induces anterior defects

Nodal signaling is required for endoderm and mesoderm formation in early frog embryos; interference with this signaling pathway hinders mesendoderm development and can lead to anterior truncation of frog tadpoles (Osada and Wright, 1999; Agius et al., 2000). BMPs are involved in ventral patterning of early frog embryos (Harland and Gerhart, 1997). If TMEFF1 regulates signals from these TGF $\beta$  ligands, then overexpression of TMEFF1 should interfere with early frog development. To examine this possibility, we injected different doses (0.5, 1, and 2 ng) of TMEFF1 RNA into either two dorsal or two ventral blastomeres of

four-cell-stage embryos. As shown in Fig. 8, while ventral expression of TMEFF1 causes tail truncation, overexpression of TMEFF1 on the dorsal side induces anterior defects in frog embryos, leading to the reduction or even absence of head structures. The cement gland and eyes are gradually lost in injected embryos. At higher doses, some embryos also show a failure in blastopore closure (not shown). Control injection of RNA encoding CD2, a membrane protein, does not lead to any embryonic defects (not shown). The morphology of the tadpoles injected with TMEFF1 RNA resembles that induced by expression of the dominant-negative nodal ligand cmXnr2 (Osada and Wright, 1999), implying that, like cmXnr2, TMEFF1 may also inhibit nodal activity in whole frog embryos. The phenotype induced by cmXnr2, however, may not be due to a complete inhibition of nodal signaling in vivo, as it has been shown that cmXnr2 still retains some of its signaling capacity, possibly through alternative processing (Eimor and Harland, 2002). In addition, cmXnr2 may also block BMP activities through heterodimerization with BMP ligands (Yeo and Whitman, 2001; Eimor and Harland, 2002). The similarity of the phenotypes between TMEFF1 and cmXnr2 embryos therefore suggests that, like cmXnr2, TMEFF1 may induce anterior defects through partial inhibition of both nodal and BMP activities.

#### Expression of TMEFF1 during early *Xenopus* development

TMEFF1 was originally identified as a gene expressed in the hypothalamo–hypophysial axis of the adult *Xenopus* brain (Eib and Martens, 1996). The expression of the gene

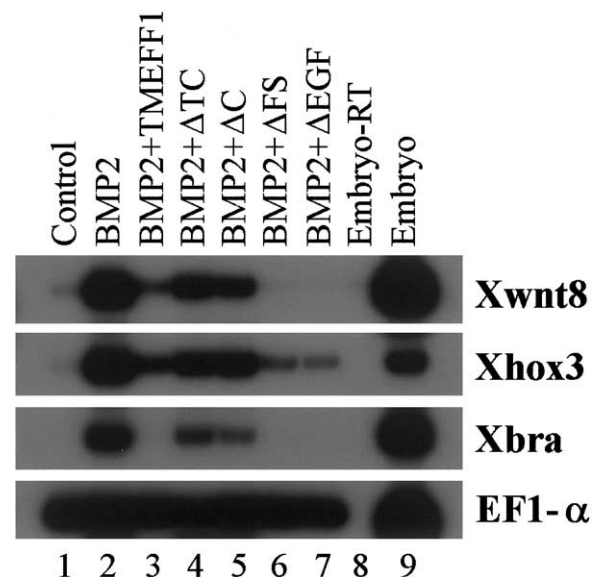


Fig. 7. Inhibition of mesoderm induction by BMP2 requires the cytoplasmic domain of TMEFF1. Deletion of the cytoplasmic tail or the transmembrane region of TMEFF1 reduces the ability of TMEFF1 to inhibit BMP2, while deletion of either the FS modules or the EGF motif does not impair the BMP inhibitory activity of TMEFF1. The doses of RNA used in this experiment are: BMP2, 0.5 ng; and TMEFF1 and the deletion mutants of TMEFF1, 1 ng.





Fig. 8. Ectopic expression of TMEFF1 interferes with anterior development of early *Xenopus* embryos. TMEFF1 RNA (0.5–2 ng) was injected into two dorsal or two ventral blastomeres of four-cell-stage embryos; and the injected embryos were analyzed at tailbud stages for morphological changes. While ventral expression of TMEFF1 leads to tail defects, dorsal expression of TMEFF1 results in reduction of anterior structures.

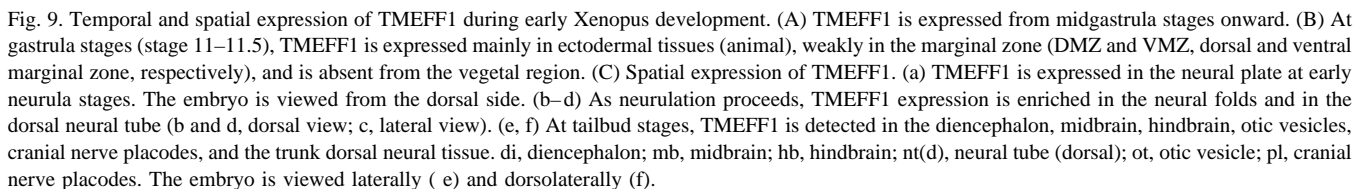
at early developmental stages in frogs has not been reported. To see when and where TMEFF1 is expressed during early frog embryogenesis, we analyzed its temporal expression profile by reverse transcription PCR (RT-PCR) and its spatial distribution by whole-mount *in situ* hybridization. As shown in Fig. 9A, TMEFF1 is first transcribed at midgastrula stages (stage 10.5). The expression level increases during neurula stages and remains at least to tadpole stages (Fig. 9A). At gastrula stages, TMEFF1 is expressed mainly in ectodermal tissues and weakly in the marginal zone, but is absent from the vegetal region (Fig. 9B). During early neurulation, TMEFF1 transcripts are detected in the neural plate (Fig. 9C, a). As neurulation proceeds, TMEFF1 RNA is restricted to the neural folds and the dorsal neural tube in the trunk region (Fig. 9C, b–d). At tailbud stages, TMEFF1 is expressed in the diencephalon, midbrain, hindbrain (Fig. 9c, f), otic vesicle, and the cranial nerve placodes (Fig. 9C, e and f) in addition to trunk dorsal neural tissue. This temporal and spatial expression pattern suggests that TMEFF1 may not participate in the early function of nodal, during germ layer formation, but may regulate TGF $\beta$  signaling during neural development.

## Discussion

During early vertebrate development, TGF $\beta$  signals regulate many aspects of embryonic induction and patterning. The activities of TGF $\beta$ s are under the control of both positive and negative factors (Hill, 2001). Follistatin, a negative regulator of both activin and BMPs, contains three repetitive modules that may be important for its inhibitory function. Proteins harboring different numbers of follistatin modules have been recently identified. FLRG, which has two FS motifs, acts similarly to follistatin in that it binds to activin with high affinity and blocks activin signaling. The

activities of Flik/FRPs, which contain single FS modules, in the regulation of TGF $\beta$  signals, however, have not been documented in detail. Here, we show that TMEFF1, a protein with two FS modules, regulates nodal and BMP, but not activin activities. This is the first example in which proteins with follistatin motifs have been shown to regulate nodal function. Our data, however, demonstrate that, though required, FS modules in TMEFF1 are not sufficient for nodal inhibition; both a second structural motif, the EGF domain, and expression of the protein at the membrane, are additionally necessary for nodal inhibition. Our results thus suggest that TMEFF1 regulates nodal activities through a novel mechanism.

How does TMEFF1 selectively block nodal but not activin signaling? In general, there are several potential mechanisms. First, TMEFF1 may bind specifically to nodal, but not activin to prevent activation of the downstream receptors, leading to selective inhibition of nodal activity. This strategy of discriminatory interaction with a subset of ligands is utilized widely for regulation of TGF $\beta$  members, by factors such as follistatin and other BMP antagonists (e.g., Zimmerman et al., 1996; Iemura et al., 1998). In the case of TMEFF1, however, a mutant protein (TMEFF1- $\Delta$ TC) that lacks the transmembrane domain and is secreted is not sufficient for nodal inhibition. The membrane localization is required for the anti-nodal effects of TMEFF1. This suggests that, if TMEFF1 does bind to nodal selectively, extracellular binding by itself is not sufficient to prevent nodal signaling. Selective binding of nodal by TMEFF1 at the cell membrane is required. A second possible mechanism is that TMEFF1 may stimulate the expression and/or activity of nodal antagonists, such as Cerberus (Piccolo et al., 1999) or Lefty (Cheng et al., 2000; Tanegashima et al., 2000), which in turn block nodal signaling. In *Xenopus*, Lefty inhibits both activin- and nodal-related



head (oep) proteins as a coreceptor for its binding to the type I receptor ALK4 for subsequent signaling (Shen and Schier, 2000; Reissmann et al., 2001; Yeo and Whitman, 2001). Cripto family members contain a divergent EGF domain and a novel CFC motif, and the CFC domain interacts directly with ALK4 to mediate nodal signal activation (Yeo and Whitman, 2001). Since TMEFF1 inhibits nodal only when it is located on the plasma membrane, it is possible that TMEFF1 interacts with Cripto proteins to prevent functional ligand–receptor complex formation in the presence of nodal. This mechanism would allow TMEFF1 to distinguish between nodal and activin. A fourth possible mechanism is that TMEFF1 activates a downstream signal or serves as a ligand to activate other signaling pathways; and the crosstalk between the TMEFF1-dependent signal and the TGF $\beta$  pathway leads to inhibition of nodal. Since secreted TMEFF1 loses its activity, it is unlikely that TMEFF1 functions as a ligand to stimulate a nodal inhibitory signal. In addition, the mutant that lacks the

cytoplasmic tail can still inhibit nodal, suggesting that signaling through TMEFF1 may not be required either. Further investigation is needed to distinguish between these possible mechanisms of TMEFF1 action.

In addition to nodal inhibition, TMEFF1 also blocks mesodermal induction by BMP2. TMEFF1 seems to achieve BMP inhibition through a distinct mechanism. A mutant that lacks the cytoplasmic domain of TMEFF1, which still retains its nodal inhibitory function, loses its ability to block BMP2. Furthermore, while deletion of either the follistatin modules or the EGF motif reduces the ability of TMEFF1 to block nodal, these deletions do not seem to affect the effectiveness of BMP inhibition. The results suggest that TMEFF1 may activate an intracellular pathway to inhibit BMP function. Interestingly, we observe that, though TMEFF1 blocks mesodermal induction by BMP2 efficiently, it does not block epidermal induction by BMP2 well, and it does not induce neural markers in animal caps. This phenomenon may be explained by several mechanisms. Much higher doses of BMP are required for mesoderm induction than for epidermal induction, and the differences of TMEFF1 in inhibition of the two inductive processes may be due to the efficiency of BMP inhibition by TMEFF1. Alternatively, the TMEFF1-activated intracellular pathway may selectively interfere with mesodermal but not epidermal patterning by BMPs. If this is the case, then the TMEFF1-dependent mechanism may work at a step downstream of the cytoplasmic signal transducer Smad1, as Smad1 is shared by both mesodermal and epidermal induction processes. Further investigations are required to understand the mechanism of BMP inhibition by TMEFF1.

In *Xenopus*, TMEFF1 RNA is not expressed in early embryos. The transcripts can be detected by RT-PCR only at gastrula stages. This temporal expression pattern differs from that seen in the mouse. During mouse development, TMEFF1 (m7365) is expressed in preimplantation embryos, and expression is maintained following implantation in both embryonic and extraembryonic tissues (De Groot et al., 2000). The difference in expression patterns of TMEFF1 therefore suggests that, unlike in mice, where TMEFF1 may regulate early nodal activity, TMEFF1 in frogs may not be involved in nodal regulation during germ layer formation and patterning. Other TMEFF homologues, including TMEFF2 (tomoregulin; Horie et al., 2000; Kanemoto et al., 2001), may exist in frogs, and they may be expressed during early frog embryogenesis to regulate nodal function in mesoderm formation. Later in development, expression of TMEFF1 in both frogs and mice is enriched in neural tissues (de Groot et al., 2000; Eib et al., 2000), suggesting that TMEFF1 may participate in modulation of nodal and BMP activities during neural patterning and differentiation.

In summary, we demonstrate in our studies that tomoregulin-1 (TMEFF1) can selectively inhibit nodal and BMP function. The membrane location of TMEFF1 is important for its nodal inhibitory activity, while the cytoplasmic region is required for BMP inhibition by TMEFF1. Our data

suggest that tomoregulin-1 may regulate nodal and BMP signaling through novel mechanisms.

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